



Phytochemical Analysis and Anti-Malarial Potential of *Eucalyptus camaldulensis* (Turare) Leaf Fractions

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ABSTRACT

Methanol extract of *Eucalyptus camaldulensis* was examined for the phytochemical compositions while the fractions were screened for anti-plasmodial potential using *in-vitro* assay method for the activity against schizonts growth of the *Plasmodium falciparum* clinical strain, the major human malaria parasite. Alkaloid, tannin, saponins, flavonoids, glycosides, steroids, terpenoids and phenols were detected. The fractions of N-hexane, ethyl-acetate and N-butanol expressed various anti-plasmodial potentials with n-hexane fraction showing highest anti-plasmodial activity against the parasite, then ethyl-acetate fraction, with IC_{50} of 0.6510 and 0.9169 $\mu\text{g/ml}$ respectively. N-butanol showed least activity with IC_{50} of 2.296 $\mu\text{g/ml}$, while the chloroquine and artemeter both of which served as positive control have IC_{50} of 0.1324 and 1.004 $\mu\text{g/ml}$ respectively. Control and normal saline served as the negative control. The anti-plasmodial activity of the fractions of the plant supported the traditional claims on its efficacy in the treatment of malaria infection.

Keywords: IC_{50} , *Plasmodium falciparum*, *in vitro*, *Eucalyptus camaldulensis* and anti-plasmodial

INTRODUCTION

Over 45% of the world's population are at risk of malaria mostly in under developed nations with over 200 million people infected with the disease annually. More than 600,000 people died annually, making malaria one of the most devastating infectious diseases worldwide with children under five years of age and pregnant women most severely affected (WHO, 2012; WHO, 2014; Mohammed *et al.*, 2014). Despite the colossal efforts put in place to eradicate the disease through drug therapies, avoidance measures and vaccine development, the rate of infection and death caused by malaria has not changed but little over the past decades (Singh, 1999). However, widespread of drug resistant strains of *Plasmodium* spp. pesticide resistant anophelid mosquitoes vector coupled with the increasing rates of poverty, population density and accelerated climatic changes have threaten the already faltering malaria eradication paradigm (Shekhawat and Vijayvergia, 2010). Four species of human malaria parasite are known which are *P. falciparum*, *P. malariae*, *P. vivax* and *P. ovale*. *P. falciparum* causes most serious form of the disease. *P. knowlesi*, a newly recognized fifth species believed to be of zoonotic origin that causes malaria in macaques monkey but can also

infect human is of significant consideration in a small geographical range in Oceania (Fong *et al.*, 1971; DFID, 2010).

The symptoms such as fever, headaches, chills, loss of appetite, vomiting, shivering, general body weakness, joint pains, dizziness and drowsiness (Brucechatt, 2005) are of important considerations, however all combined may not present in a single patient and if they do, the severity varies from individuals. Confirmation is usually done by laboratory examination of blood for the presence of parasites (Avwioro, 2010). Complication of the disease include childhood deaths, anaemia, low birth weight, epilepsy and neurological problems (Cox -Singh *et al.*, 2008). Ninety percent (90%) of malaria cases in the world are estimated to be in Africa where the disease is endemic particularly west and central. (WHO, 2008; Kachur *et al.*, 2010) (DFID, 2010). It account for 40% of public health expenditure, 30-50% of inpatient admissions, and up to 50% outpatient visits in area with high malaria transmission (Ngemenya *et al.*, 2006). Dexter, 2012)

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Measures for containment are urgently needed to limit the spread of drug -resistance and to prevent a major threat to current plans for eliminating malaria through the search for antimalarial among local herbs such as *E. camaldulensis*. This work is therefore designed to examine the phytochemical composition and anti-plasmodial potential of the methanol crude extract and the n- hexane, ethylacetate and n- butanol fractions of the leaf of *E. camaldulensis* plant.

METHODOLOGY

Plant materials and Parasite

The plant material used for the research is *E. camaldulensis* leaf. While *P. falciparum* clinical strain was used for the anti-plasmodial screening of the plant extract and fractions.

Sample collection

Fresh *E. camaldulensis* leaves was collected from the biological garden of Applied Biology Department Kaduna Polytechnic. The plant was identified by a Botanist in the Herbarium unit of the Department of Botany, Ahmadu Bello University Zaria and Sample specimen with voucher number VN 2510 was deposited at the herbarium of the Department.

Sample Preparation

Fresh leaf of *E. camaldulensis* was washed with water, cut into pieces and air dried at room temperature (25⁰C) for two weeks. The dried leaf was then pulverized using ceramic mortar and pestle. The powdered was weighed weighing and stored in a clean polyethylene bags until required for further analysis.

Extraction of Plant Samples

Fifty two (52g) of the powder was weighed into the extraction container and 200ml of methanol was added and covered, the mixture was left for 48hours with intermittent shaking. The mixture was filtered using Whatman filter paper and concentrated on rotary evaporation under reduced pressure at 35⁰C and stored dried for further use. The percentage yield of the crude extract was calculated using the following formula.

$$\text{Percentage Yield} = (\text{Weight of Crude extract}) / (\text{Total weight of the Sample}) \times 100$$

Phytochemical analysis

The crude leaf extract of the plant was analyzed for phytochemicals by routine standard procedures as described by

Sofowara, (1993) and Trease and Evans (1996).

Fractionation of the Crude Extract

About 2g of concentrated methanol extract of the *E. camaldulensis* was dissolved in 100ml distilled water in a 250ml flask. The solution was then partitioned using solvents of varying polarity from non-polar to highly polar (n-hexane, ethyl acetate and n-butanol respectively). The extract was poured into separating funnel, equal volumes of the aqueous extract solution and solvent to be used for partitioning were poured into the separating funnel. The funnel was close with a stop cock and was gently shaken by inverting and swirling several times. The mixture was then left to separate into two distinct phases. The stop cock was removed and the tap was opened to release the bottom layer into a clean and dried beaker. The top layer was poured out into another beaker through the top opening of the separating funnel. Each of the fractions obtained was evaporated at room temperature. Each of the concentration of the fractions was kept in a weighed and labelled container until required for use.

In vitro Anti-plasmodial Assay

The malaria culture medium for cultivating of *P. falciparum* was prepared from one packet of RPMI1640 (containing 25mm of HEPES, buffer glucose dissolved in 960ml of double distilled water, 40mg of gentamicin sulphate (1.2ml of gentamicin) was added. This solution was passed through a Millipore filter paper of 0.22 μ m and stored as 4⁰C as 96ml aliquot in glass media bottle.

Preparation of Washing Medium

Eight grams (8 grams) of sodium chloride, 0.2grams of potassium chloride, 0.24 grams of di-potassium hydrogen phosphate and 1.4 grams of di-sodium hydrogen phosphate were all dissolved and make upto 100ml double distilled water and filtered through a Millipore filter paper of 0.22 μ m and stored as 4⁰C.

Serum Preparation

A sample of blood was collected into a plan bottle and kept at 4⁰C for 24hours, centrifuged at 10,000rpm for 20minutes. Serum was separated aseptically and kept into a liquots. The serum was inactivated by keeping it 56⁰C water bath for half an hour.

Preparation of Erythrocyte (RBCs) for Culture

A sample O⁺ blood was collected in anticoagulant tubes. The sample was then centrifuged at 1,500rpm for 10minutes at room temperature. Plasma and buffy coat was removed with sterile Pasteur pipette after which washing media was added, centrifuge was repeated thrice equal amount of complete was added to the pallet and stored as 4^oC. A suspension of infected cells (50%) with complete media (with 15% de-activated serum) was prepared. Appropriate amount of uninfected red blood cell was added to get an initial parasitemia of 0.5% to 1.0% and diluted with complete media to get 5% cell suspension (5% hematocrit). The culture was kept in kindle jar at 37^oC.

After every 24hours the media was removed using a sterile Pasteur pipette without disturbing the cells that settled. The cells were mixed without frothing and a drop of blood was placed on the slide and a thin film was made.

Fresh complete media (with 10% serum) was added, missed properly, and kept back into the kindle jar. Thin film was stained and examined for parasitemia.

A blood drop (3.0 μ l) was placed on a slide toward left side. The cell were smeared across the slides with help of another slide to get a thin film and dried at room temperature. The slides were fixed by immersing in methanol for 30 second followed by drying. The slides were then stained with giemsa stain for 15 seconds and washed with water to removed excess stain. It was then observed under the microscope. Estimation of the percentage of erythrocytes infected (1% parasitemia) with *P. falciparum*. An area of stained thin blood film where the erythrocytes are distributed was observed using x 100 objectives (under oil immersion).

Total number of red blood cell in area was counted by moving the slide, the number of infected erythrocyte among the total red blood cell was also counted. The slide was removed randomly to adjacent field and counting continued as mention above. Percentage parasitemia is calculated by dividing total number of parasitized cell in the three fields by total number of red blood cell in the three fields x 100 (Neelam and Nilofer, 2010).

% parasitemia =

$$\frac{\text{Number of infected blood cell}}{\text{total number of the red blood cell}} \times 100$$

Synchronization of *P. falciparum*

Sorbitol (5%) was prepared in distilled water and passed through a Millipore filter paper of 0.22 μ m porosity. To 1ml of the culture pallet, 9ml of sorbitol was added and kept at room temperature for 5minutes. The supernatant was removed by centrifugation and pallet was washed thrice in complete media (washing procedure same as mention for preparation of erythrocytes for culture). Freshly washed erythrocytes were added to the culture.

Schizonts Growth Inhibition Activity and IC₅₀ Determination

The test was performed in triplicate in a 96 well micro-titre plate that is based on assessing the inhibition of schizont maturation while, RPMI 1640 (sigma company U.S.A) was the culture medium used. Serial dilutions were made from the various fractions of plant extract with concentration (100, 50, 25, 12.5, 6.75, 3.35, and 1.75 μ g/ml) respectively. Negative control was treated with the solvent and positive controls by chloroquine phosphate and Artemether) was added to each set of experiments. 50 μ L from blood mixture media was added to the wells in plate and incubated at 37^oc for 24hours. After incubation, content of the wells was harvested and stained for 40minutes in 10% glemsa solution pH 7.2. The count process was done in triplicate, parasite count for each blood film was done. Each film was observed three different visual filed the number of schizont with three or more nuclei per 200 parasites was counted. Growth inhibition was expressed as the percentage of schizonts in each concentration compared with controls. Growth in the drug treated groups was calculated as follows:

$$\text{Percentage inhibition} = \frac{\text{Parasitemia in control well} - \frac{\% \text{ parasitemia in test well}}{\% \text{ parasitemia in the control well}} \times 100}{\% \text{ parasitemia in the control well}} \times 100$$

The number of schizonts observed per well was entered into nonlinear regression software which was specific for the analysis of in-vitro drug sensitivity assay for malaria. Individual dose response curves were generated and their IC₅₀ values were determined.

RESULTS

Percentage yield and organoleptic properties of crude extract and the fractions of *E. camaldulensis*

The crude extraction process was undertaken using methanol solvent and the result as represented in table 1 indicated the 4.1% yield. However the liquid –liquid fractionation showed n-butanol has the highest yield with 2.97% while ethyl-acetate has the least with 0.93% n-hexene was 1.62%. the crude extract, n-hexene and n-butanol are dark green ethyl-acetate has a lemon green colour. The fractions are powdery only n-butanol is gummy, while they general have sweet, fruity smell n-butanol fraction is practically odourless (Table 1).

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Table 1: Percentage yield and organoleptic properties of methanol and fractions of *E. camaldulensis*

Extract/ Fraction	Percentage yield (%)	Organoleptic properties		
		Colour	Texture	odour
Methanol	4.1%	Dark green	Oily	Sweet
n-hexane	1.62%	Dark green	Powdery	Petrollic
Ethylacetate	0.93%	Lemon green	Powder	Fruity
n-butanol	2.97%	Dark green	Gummy	Odorless

Phytochemical Composition of methanol crude leaf extract of *E. camaldulensis*

The results showed that the phytochemical compositions detected in the crude extract includes Alkaloids, tannin, saponins, flavonoids, glycosides, steroids, terpenoids and phenols (Table 2), while anthraquinones and glycosides tested were not detected.

Table 2: Phytochemical Analysis of methanol crude leaf extract of *E. camaldulensis*

Phytochemicals	
Alkaloids	+++
Amino Acids	+
Anthraquinones	-
Carbohydrates	++
Flavonoids	+++
Glycosides	-
Phenols	+++
Saponins	+++
Tanins	++
Terpenoids	++
Steroids	++
Triterpenes	+

+ = Present

+++ = highly intensive

++ = intensive

- = Not present

Average Percentage inhibition and IC₅₀ of Different Fractions of *E. camaldulensis*

The in-vitro anti-plasmodial assay results indicated Ethyl-acetate have higher activity against the schizont maturation profile (Table 3) with the least IC₅₀ of 0.6510µg/ml (Table 4) while chloroquine indicated the highest profile among the control with IC₅₀ 0.1325µg/ml, n-butanol indicated the least percentage inhibition activity and with IC₅₀ of 2.296 µg/ml (Table 3, and 4)

Table 3: Average Percentage inhibition of Different Fractions of *E. camaldulensis* chloroquine and Artemeter

Fractions	Concentrations in $\mu\text{g/ml}$						
	1.75	3.35	6.75	12.8	25	50	100
	Average % inhibition						
n-hexane	57.96	60.32	71.99	75.98	78.94	82.44	89.67
Ethylacetate	67.88	81.55	87.25	91.52	93.82	96.46	97.36
N-Butanol	45.39	56.16	75.49	85.16	88.82	99.06	99.49
Artemether	59.47	70.24	80.72	85.31	90.09	94.45	96.22
Chloroquine	82.42	86.89	92.16	93.59	94.40	97.34	99.26

Table 4: IC_{50} of fraction of *E. camaldulensis*, chloroquine and Artemeter

Fractions	$IC_{50}/\mu\text{g/ml}$
n-hexane	0.9169
ethylacetate	0.6510
n-butanol	2.296
Artemether	1.004
Chloroquine	0.1324

DISCUSSION

Medicinal plants are widely used in Africa communities for the treatment of different types of bacterial fungal and parasitic diseases. *E. camaldulensis*, been an Australian native, is a genus of tall, evergreen and magnificent trees cultivated all over the world for its oil, gum, pulp, timber, medicine and aesthetic value. Its essential oil are found in abundance in its foliage. In addition, the oil possesses a wide spectrum of biological activity which include anti-microbial, fungicidal, insecticidal/insect repellent, herbicidal, acaricidal and nematocidal. Crude methanol extract of *E. camadulensis* has been reported to inhibit the growth of *Candida albicans* (Babayi *et al.*, 2004)

This is directly linked to the rich composition of a wide variety of secondary metabolites, with anti-activities properties (Sher, 2009). The percentage yields of the methanol extract, N-hexane, ethylacetate and N-butanol fraction of *E. camadulensis* were generally low. However the alcohol is believed to be the best organic extractor, which is portrayed in the *anti-plasmodial* potency demonstrated by all the fractions. The organoleptic properties of the extract and fraction indicated that the methanol extract was dark green in colour, oily in texture and sweet smell.

The fractions of *E. camadulensis* plant were tested on the schizonts of *Plasmodium falciparum* as IC_{50} (concentration at which the inhibition of the parasite growth presence (50%) with the chloroquine and arthemether as positive controls. The claim by traditional healers of the plant potential as antimalarial agent was confirmed by this research work with all the fractions showing percentages of inhibition of the *P. falciparum* clinical strain between 56 to 99% which highly demonstrated *anti plasmodial* potential. Since all extracts with potential to reduce parasitamea level above 30% is considered active (Vale *et al.*, 2015). On the other hand the IC_{50} value of ethyl-acetate, N-hexane and N-butanol fractions demonstrates very high potential with decrease in growth of parasite with IC_{50} of 0.9169, 0.6510 and 2.29 $\mu\text{g/ml}$ which is a very remarkable potential and compare favourably with the positive controls, chloroquine and arthemether with IC_{50} of 0.1324 and 1.004 $\mu\text{g/m}$ respectively, even though the extracts are only partially purified. All of the drugs showed very good *anti-plasmodial* activity with IC_{50} considered as very highly effective.. Accordingly antimalarial activity for plants, based on the best laboratory practices compounds/extracts are generally considered as strongly active if the $IC_{50} < 5 \mu\text{g/ml}$ (Chinchilla *et al.*, 2012; Sha'a *et al.*, 2014). The presence of alkaloids, tanins, saponins, flavanoids, glycosides, steroids and phenols detected in the phytochemical screening of the crude extract could be related to the antiplasmodial activity plant as reported (Singab *et al.*, 2011; Osuagwu *et al.*, 2007)

Conclusion

The invitro *anti-plasmodial* activity of *E. camelendulensis* was demonstrated with three fractions of n-hexane, ethylacetate and n-butanol respectively, and all the fractions

showed significant *anti-plasmodial* activity with n-butanol having the least *anti-plasmodial* activity with IC₅₀ of 2.296ug/ml n-hexane and ethylacetate showed higher activity with IC₅₀ value of 0.916ug/ml and 0.6510ug/ml respectively which could be considered as a better effective solvent for their purification of the lead compound.

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